

# (12) UK Patent Application (19) GB (11) 2 240 100 (13) A

(43) Date of A publication 24.07.1991

(21) Application No 9001088.5

(22) Date of filing 17.01.1990

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(51) INT CL<sup>6</sup>  
C07D 498/04, A61K 31/535, C12P 17/18 //  
(C07D 498/04 207:16 265:02) (C12P 17/18 C12R  
1:845)

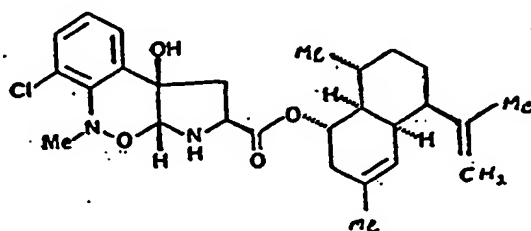
(52) UK CL (Edition K)  
C2C CAA CBC C136X C200 C213 C214 C22Y C22Z  
C25Y C255 C306 C31Y C313 C337 C36Y C360  
C363 C366 C368 C623 C628 C66X C672 C678  
C80Y C801 C805  
C8Y Y337 Y501 Y502  
U1S S1308 S1312 S2410

(56) Documents cited  
None

(58) Field of search  
UK CL (Edition K) C2C CBC  
INT CL<sup>6</sup> C07D  
Online database : CAS ONLINE

(54) Anti-parasitic compound

(57) An antiparasitic compound of formula (I) is prepared by fermentation of micro-organism *Chrysosporium* sp N845-98 (ATCC 20975).



(I)

**1**

Thus one aspect of the present invention provides compound UK-88051 having the formula (I) as determined by x-ray crystallography.



Compound UK-88051 is produced by the submerged aerobic fermentation in aqueous nutrient media or by a solid agar fermentation under aerobic conditions of a microorganism isolated from a soil sample collected in Ushimado Town, Okayama Prefecture, Japan. This microorganism has been deposited in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA under the accession number ATCC 20975 on 22 December

1989. It is designated herein as culture N845-98. It has been characterised as follows:

The culture N845-98 was three-spot inoculated from a potato dextrose agar slant onto plates of identification media and the plates were incubated at 28°C for two to three weeks. The colours were determined by comparisons with colour chips from Color Standards and Color Nomenclature by Robert Ridgway, 1912.

Identification media used for the characterization of the culture and references for their composition are as follows:

Cornmeal Agar - Carmichael, J. W. 1957. Mycologia 49: 820-830.

Czapek-Sucrose Agar - Raper, K. B. and D. I. Fennell. 1965. The Genus Aspergillus, p.36.

Malt Extract Agar - Ibid, p.38.

Oatmeal Agar - ISP #3 medium, Difco.

Yeast Extract-Soluble Starch Agar - Emerson, R. 1958. Mycologia 50: 589-621.

Czapek-Sucrose Agar - Colonies attaining 2 cm diam. in 16 days; pale brownish drab to light brownish drab (XLV) at the centre, white to off-white toward the edge; raised at the centre, thin or submerged toward the edge, smooth; velvet, aerial mycelium white to off-white; reverse maize yellow (IV); no soluble pigment; conidial production poor.

Oatmeal Agar - Colonies attaining 2.2 cm diam. in 16 days; pallid brownish drab (XLV), pale mouse gray to light mouse gray (LI); slightly raised, smooth; velvet to slightly cottony, aerial mycelium same as surface; with hyaline exudate; reverse hellebore green (XVII) or darker; no soluble pigment; conidial production good.

Yeast Extract-Soluble Starch Agar - Colonies attaining 2.6 cm diam. in 16 days; pale brownish drab to light brownish drab (XLV); raised but thin toward edge; coarsely and radiately wrinkled, smooth toward edge; velvet, aerial mycelium same as surface; with hyaline exudate; reverse black to benzo brown (XLVI), cream toward edge; with yellow soluble pigment; conidial production good.

Corrmeal Agar - Colonies attaining 2.0 cm diam. in 16 days; white to black; raised but thinner toward edge, smooth; velvet, aerial mycelium white; with hyaline exudate; reverse hellebore green (XVII) or darker, rinnemann's green (XVIII) to cource green (XVII) toward edge; with javel green (V) soluble pigment; conidial production abundant. Vegetative hyphae septate, 1.5-3.0  $\mu$ m wide. Sporulating hyphae undifferentiated, pale brown, smooth, 1.2 to 2.0  $\mu$ m diam, producing short conidiophores upon which conidia are produced. Conidiophores short and narrow. Conidia one-celled; pale brown, pale grayish brown with age, dark brown in mass; sessile or on short conidiophores, produced singly or rarely in short chains, aleuriosporic; elliptical to pyriform, often truncate at the base, 2.5-4.0 x 1.8-3.0  $\mu$ m; coarsely roughened, as observed under the light microscope.

Malt Extract Agar - Colonies attaining 1.5 cm diam. in 16 days; white to off-white, but sulphur yellow (V) to pinard yellow (IV) at centre; moderately raised at centre, thin or submerged toward edge; smooth to occasionally radiately wrinkled; velvet, aerial mycelium same as surface; reverse wood brown to natal brown (XL), pale yellow to cream toward edge; no soluble pigment; conidial production moderate. Vegetative hyphae septate, 1.5-3.5  $\mu$ m wide. Chlamydospores terminal or intercalary, single or contiguous,

thin- or thick-walled, hyaline to pale brown; globose, subglobose, oval to elliptical; 3-7  $\mu\text{m}$  diam or 2.5-9.0 x 3.0-7.0  $\mu\text{m}$ .

Sporulating hyphae undifferentiated, hyaline to pale brown, smooth, 1.2 to 3.0  $\mu\text{m}$  diam, producing short conidiophores upon which conidia are produced. Conidiophores, if present, short, smooth, hyaline to pale brown, up to 5  $\mu\text{m}$  long and 1.2 to 2.0  $\mu\text{m}$  wide. Conidia one-celled; pale brown, pale grayish brown with age, dark brown in mass; sessile or on short conidiophores, produced singly or rarely in short chains, aleuriosporic; ovoid, elliptical to pyriform, often with a truncate base, 2.5-4.0 x 2.0-3.0  $\mu\text{m}$ ; appearing coarsely roughened by light microscopy but warty by scanning electron microscope.

The culture N845-98 is characterized by the white, yellowish, pale brownish drab to pale mouse gray colonies; the yellowish, greenish to brownish colony reverse; the pale brown, aleuriosporic conidia which are warty and with a truncate base. These features fit into the definition of the genus Chrysosporium Corda, as defined by Carmichael (Carmichael, J. W., Can. J. Bot. 40: 1137-1173, 1962) and by Van Oorschot (Oorschot, C. A. N. van., Studies in Mycology, No. 20, 89 pp., 1980).

The culture N845-98 closely resembles Chrysosporium lobatum Scharapov in the colours of colonies and the size and shape of pigmented conidia, but differs in the absence of intercalary conidia, the lack of orthotropic branching of the fertile hyphae, and the warty rather than echinulate conidia. Thus, the culture N845-98 is considered as a species of the genus Chrysosporium and designated as Chrysosporium sp.

Cultivation and isolation of compound UK-88051 may be

conducted under conditions similar to those generally employed to produce antibiotics by fermentation. Cultivation may take place in an aqueous nutrient medium containing suitable sources of carbon, nitrogen and trace elements, for a period of several days under aerobic conditions at a temperature in the range of 24 to 36°C. As with the majority of antibiotic fermentations the amount of UK-88051 will vary with changing fermentation conditions especially with regard to nutrient components, aeration conditions and pH. The mycelial product is then recovered by centrifugation or filtration and extracted with acetone or methanol. The solvent extract is concentrated and either freeze-dried or the desired products may be extracted into a water-immiscible organic solvent, such as methylene chloride, hexane, petroleum spirit, ethyl acetate, chloroform, butanol or methylisobutyl ketone. The solvent extract is concentrated and the crude product is further purified as necessary by chromatography. Final purification of UK-88051 can be achieved by repeated column chromatography or using a technique such as reverse phase high pressure liquid chromatography (HPLC), or by preparative thin layer chromatography.

Alternatively, cultivation may take place on agar plates of a suitable medium under aerobic conditions at a temperature in the range of 24 to 36°C for several days. The agar with the mycelial growth is then extracted with an organic solvent such as methanol, filtered and the filtrate concentrated. Further enrichment and separation of UK-88051 is then carried out as described above.

Thus another aspect of the present invention provides a process for producing a compound designated UK-88051 which

comprises cultivating the microorganism N845-98, or a mutant, genetically transformed or recombinant form thereof having the ability to produce said compound, in submerged aqueous or solid agar culture media containing an assimilable source of carbon, nitrogen and inorganic salts, under aerobic fermentation conditions until a recoverable amount of said compound is obtained.

The term mutant includes any mutant strain which arises spontaneously or by the application of known techniques, such as exposure to ionising radiation, ultraviolet light, and/or chemical mutagens such as N-methyl-N-nitroso-urethane, N-methyl-N'-nitro-N-nitrosoguanidine and ethane methane sulphate, etc. Genetically transformed and recombinant forms include mutants and genetic variants produced by genetic engineering techniques, including for example recombination, transformation, transduction, and protoplast fusion, etc. The invention also extends to UK-88051 produced by said process.

As previously mentioned the compound of the invention possesses significant antiparasitic activity having particular utility as an anthelmintic, ectoparasiticide, insecticide and acaricide.

Thus UK-88051 is effective in treating a variety of conditions caused by endoparasites including, in particular, helminthiasis which is most frequently caused by a group of parasitic worms described as nematodes and which can cause severe economic losses in swine, sheep, horses and cattle as well as affecting domestic animals and poultry. The compound is also effective against other nematodes which affect various species of

animals including, for example, Dirofilaria in dogs and various parasites which can infect humans including gastro-intestinal parasites such as Ancylostoma, Necator, Ascaris, Strongyloides, Trichinella, Capillaria, Trichuris, Enterobius and parasites which are found in the blood or other tissues and organs such as filarial worms and the extra intestinal stages of Strongyloides and Trichinella.

The compound is also of value in treating ectoparasite infections including in particular arthropod ectoparasites of animals and birds such as ticks, mites, lice, fleas, blowfly, biting insects and migrating dipterous larvae which can affect cattle and horses.

The compound is also an insecticide active against household pests such as the cockroach, clothes moth, carpet beetle and the housefly as well as being useful against insect pests of stored grain and of agricultural plants such as spider mites, aphids, caterpillars and against migratory orthopterans such as locusts.

The compound UK-88051 may be administered as a formulation appropriate to the specific use envisaged and to the particular species of host and animal being treated and the parasite or insect involved. For use as an anthelmintic the compound may be administered orally in the form of a capsule, bolus, tablet or liquid drench, or alternatively, it may be administered as a pour-on or by injection, either subcutaneously or intramuscularly. Capsules, boluses or tablets may be prepared by mixing the active ingredient with a suitable finely divided diluent or carrier, additionally containing a disintegrating agent and/or binder such as starch, lactose, talc, or magnesium stearate. A drench



formulation may be prepared by dispersing the active ingredient in an aqueous solution together with dispersing or wetting agents and injectable formulations may be prepared in the form of a sterile solution or emulsion.

Pour-on and injection formulations are prepared in a conventional manner in accordance with standard veterinary practice. These formulations will vary with regard to the weight of active compound depending on the species of host animal to be treated, depending on the severity and type of infection and the body weight of the host. Generally for oral administration a dose of from about 1 to 100 mg per Kg of animal body weight given as a single dose or in divided doses for a period of from 1 to 5 days will be satisfactory but of course there can be instances where higher or lower dosage ranges are indicated and such are within the scope of this invention.

As an alternative the compound may be administered with the animal feedstuff and for this purpose a concentrated feed additive or premix may be prepared for mixing with the normal animal feed.

For use as an insecticide and for treating agricultural pests the compound is applied as sprays, dusts, pour-on formulations, emulsions and the like in accordance with standard agricultural practice.

For human use the compound is administered as a pharmaceutically acceptable formulation in accordance with normal medical practice.

The invention is illustrated by the following Examples in which Example 1 describes the preparation, isolation and identification of UK-88051 and Example 2 describes the

anthelmintic activity of UK-88051.

In Example 1, Oxoid peptone, Oxoid Lab Lemco and Oxoid Yeast Extract were supplied by Oxoid Limited, Wade Road, Basingstoke, Hampshire, U.K.

Ultraviolet spectra were recorded using a Hewlett Packard 8452A diode array spectrophotometer.

Fast atom bombardment mass spectroscopy was performed using a VG model 7070E mass spectrometer. Samples were introduced using a matrix consisting of glycerol, thioglycerol and trichloroacetic acid.

Nuclear magnetic resonance spectral data were obtained using a General Electric GN500 spectrometer or a Nicolet GE 300 spectrometer.

Example 1

The microorganism N845-98 was maintained on a slant of the following composition:

	g/litre
Glucose	10
Starch	20
Bactocasitone	5
Difco Yeast Extract	5
Calcium carbonate	1
Difco Agar	20
pH	7.1

A well-grown slant of the organism N845-98 on the above medium was rubbed off with 10 ml of sterile distilled water. 5 ml aliquots of this suspension were used to inoculate two 300 ml Erlenmeyer flasks each containing 50 ml of a medium of the following composition:

	g/litre
Glucose	1
Starch	24
Oxoid Peptone	5
Oxoid Yeast Extract	5
Oxoid Lab Lemco	3
Calcium Carbonate	4
pH 6.8-7.0	

These were incubated at 28°C on a rotary shaker with a 1 inch (2.54 cm) throw operating at 180 rpm. After 2 days the contents of each flask were transferred to two 3 litre Fernbach flasks each containing 1 litre of the same medium and incubation was continued as above for 48 hours.

These flasks were used to inoculate a New Brunswick 100 litre fermenter containing 100 litres of a medium of the following composition:

	g/litre
Glucose	5
Bacto-casitone	2.5
Trusoy Flour	2.5
Starch	10
Yesta yeast extract	2.5
Calcium carbonate	2
Cobalt chloride	0.005
pH 7.0	

This fermentation was operated at 28°C with an agitation speed of 150 rpm and an aeration rate of 60 litres per minute. After 12 days the mycelium was separated by filtration and extracted with methanol. The spent mycelium was removed by filtration and the methanol solution concentrated to low volume under vacuum and finally freeze dried to give a dark brown gum (60 g). The bulk of this material (54.2 g) was agitated with methanol (1.5 litres) in an ultrasonic bath and the suspension was filtered through Hyflo. The filtrate was evaporated to 500 ml under

vacuum, filtered again and concentrated to dryness. The residue was agitated with petroleum spirit (bp 40–60°C) (300 ml x 3) and the combined extracts were concentrated to dryness under vacuum. This crude residue (14.2 g) was chromatographed on silica (Merck Rieselgel 60, mesh 230–400) eluting initially with dichloromethane - ethyl acetate 4:1 then 2:1, 1:1 by volume and finally with pure ethyl acetate. Fractions were analysed for UK-88051 by hplc using an Ultrasphere ODS (Trademark, Beckman) column (4 x 250 mm) eluting with a water-methanol gradient starting at 20:80 and finishing at 5:95 over 40 minutes at 0.85 ml per minute. The desired component eluted under these conditions after 20 minutes. Fractions containing UK-88051 were combined and final purification was achieved by semi-preparative hplc using an Ultrasphere-ODS (Trademark, Beckman) column (10 x 250 mm) eluting with a water-methanol mixture (1:4) at 3 ml per minute. Fractions containing pure UK-88051 eluted at around 60 minutes and were combined and evaporated to give a white powder, mp 112–114°C with the following characteristic spectroscopic properties:

a) Mass spectrum (FAB)  $m/e = 487$  ( $M + M^+$ ) calculated for  $C_{27}H_{35}N_2O_4Cl.H^+ = 487$ .

b) Ultraviolet absorption spectrum

217	( $\epsilon = 17,530$ )
248	( $\epsilon = 4,301$ )
287	( $\epsilon = 1,389$ )

c) Carbon-13 magnetic resonance spectrum ( $CDCl_3$ ) signals downfield from TMS at

$\delta$	175.5	(s)
	146.56	(s)
	143.04	(s)
	131.07	(s)

- 13 -

130.12	(s)
129.10	(d)
125.10	(d)
123.99	(d)
122.76	(s)
120.22	(d)
109.87	(t)
84.78	(d)
75.55	(s)
72.24	(d)
57.38	(d)
45.92	(d)
44.38	(d)
41.84	(q)
41.64	(t)
34.80	(t)
31.37	(unresolved d + t)
28.70	(d)
25.33	(t)
22.67	(q)
22.03	(q)
19.04	(q)

-14-

d) Proton magnetic resonance spectrum ( $\text{CDCl}_3$ ):

7.5	(d, 1H)
7.25	(d, 1H)
7.05	(t, 1H)
5.37	(m, 1H)
5.19	(bs, 1H)
5.15	(bs, 1H)
4.92	(m, 1H)
4.70	(bs, 1H)
4.07	(dm, 1H)
3.66	(bs, 1H)
3.32	(s, 3H)
3.19	(bs, 1H)
2.77	(bs, 1H)
2.51	(dd, 1H)
2.32	(d, 1H)
2.27	(dm, 1H)
2.02-1.95	(m, 2H)
1.78	(s, 3H)
1.69	(dq, 1H)
1.64	(bs, 3H)
1.62	(m, 1H)
1.55	(dm, 1H)
1.32	(qd, 1H)
1.27	(m, 1H)
1.05	(qd, 1H)
0.98	(d, 3H)

Example 2

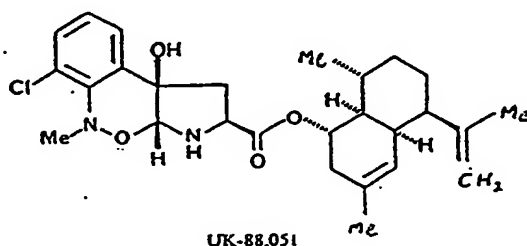
Anthelmintic activity of the compound obtained as in Example 1 was evaluated against Caenorhabditis elegans using the in vitro screening test described by K. G. Simpkin and G. L. Coles in Parasitology, 1979, 79, 19. The compound killed 95% of the worms at a well concentration of 5 parts per million.



-16-

Claims

1. A composition having antiparasitic properties obtainable by fermentation of micro-organism N845-98 or a mutant or recombinant form thereof having the ability to produce said composition.
2. A process for producing a composition having antiparasitic properties which comprises fermenting micro-organism N845-98 or a mutant or recombinant form thereof having the ability to produce said composition in a fermentation medium, and isolating said composition from the medium.
3. A compound of formula (I):



(I)

4. A compound obtainable by fermentation of micro-organism N845-98 having the following spectroscopic characteristics (a) to (d):
- a) Mass spectrum (FAB)  $m/e = 487$  ( $M + M^+$ ) calculated for  $C_{27}H_{35}N_2O_4Cl \cdot H^+ = 487$ ).
  - b) Ultraviolet absorption spectrum
 

217	( $\epsilon = 17,530$ )
248	( $\epsilon = 4,301$ )
287	( $\epsilon = 1,389$ )

-17-

c) Carbon-13 magnetic resonance spectrum ( $\text{CDCl}_3$ ) signals

downfield from TMS at	$\delta$	175.5	(s)
		146.56	(s)
		143.04	(s)
		131.07	(s)
		130.12	(s)
		129.10	(d)
		125.10	(d)
		123.99	(d)
		122.76	(s)
		120.22	(d)
		109.87	(t)
		84.78	(d)
		75.55	(s)
		72.24	(d)
		57.38	(d)
		45.92	(d)
		44.38	(d)
		41.84	(q)
		41.64	(t)
		34.80	(t)
		31.37	(unresolved d + t)
		28.70	(d)
		25.33	(t)
		22.67	(q)
		22.03	(q)
		19.04	(q)

-18-

d) Proton magnetic resonance spectrum ( $\text{CDCl}_3$ );

7.5	(d, 1H)
7.25	(d, 1H)
7.05	(t, 1H)
5.37	(m, 1H)
5.19	(bs, 1H)
5.15	(bs, 1H)
4.92	(m, 1H)
4.70	(bs, 1H)
4.07	(dm, 1H)
3.66	(bs, 1H)
3.32	(s, 3H)
3.19	(bs, 1H)
2.77	(bs, 1H)
2.51	(dd, 1H)
2.32	(d, 1H)
2.27	(dm, 1H)
2.02-1.95	(m, 2H)
1.78	(s, 3H)
1.69	(dq, 1H)
1.64	(bs, 3H)
1.62	(m, 1H)
1.55	(dm, 1H)
1.32	(qd, 1H)
1.27	(m, 1H)
1.05	(qd, 1H)
0.98	(d, 3H)

5. An antiparasitic composition, comprising a compound according to claim 3 or 4.
6. A compound according to claim 3 or 4, for human or veterinary medicine.
7. Use of a compound according to claim 3 or 4, for making a medicament for treatment of parasitic diseases.
8. An antiparasitic compound or composition, substantially as herein before described with reference to the Examples.
9. Micro-organism Chrysosporium sp N845-98.